Biosynthetic Study of Alternaric Acid: Isolation of Plausible Biosynthetic Intermediates and Origins of the Hydrogen and Oxygen Atoms

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In further isolation studies of alternaric acids, new less-oxidized analogues, (10*E*)-10,11-dideoxy-10,11-dehydro-6,19-dihydroalternaric acid **5** and 10,11-dideoxy-6,19-dihydroalternaric acid **6**, have been isolated from *Alternaria solani*, which is a causal fungus of early blight disease on potato and tomato. The structures have been elucidated by spectroscopic analysis. HPLC analysis of the acidic extracts of the cultural filtrates which had been treated with specific cytochrome P-450 inhibitors were employed, and also incorporation of sodium [1-^{13}C,$^{18}O_2$]- and [1-^{13}C,$^{2}H_3$]-acetate into the metabolites have been carried out. In addition, treatment of the fungus with the inhibitors resulted in the generation of a plausible precursor, which we have named proalternaric acid I **7**. The structure and stereochemistry of **7** have been determined by spectroscopic analysis and chemical synthesis from the analogue **3**. From the results of these experiments, plausible biosynthetic routes to alternaric acid **1** are postulated.

Alternaric acid **1** was isolated in 1949 by Brian *et al.* from *Alternaria solani*, which is a causal fungus of early blight disease on potato and tomato, as an antifungal metabolite.[1a] After that, it was shown to contribute to disease development in the host by *A. solani* in a manner similar to the mode of action of the group of compounds classified as host-specific toxins, although all of the requirements as a primary disease determinant were not fulfilled.[2] This phytotoxin **1** was also shown to delay the occurrence of hypersensitive death of potato cells infected by an incompatible race of *Phytophthora infestans*.[3] Recently, we disclosed the determination of the complete stereochemistry, and achieved a total synthesis, of alternaric acid **1**.[4] Moreover, we presented further work on the isolation, structure elucidation, and determination of absolute configurations of three new metabolites **2**, **3** and **4** related to compound **1**.[5] Although biosynthetic studies of alternaric acid building units have been carried out,[6] the late biosynthetic route has not been investigated. For the purposes of this investigation, we describe herein the structure elucidation of two new metabolites **5** and **6**, related to alternaric acid **1**, and we report P-450 inhibitor experiments as well as feeding experiments carried out to obtain pertinent data.

Cytochrome P-450 is a well known oxidative enzyme located in numerous organisms, from bacteria to mammals,[7] which plays an important role in the biosynthesis of steroids[7b] and the plant hormone gibberellin.[8] Therefore, a variety of P-450 inhibitors have been developed as clinical drugs and plant growth regulators.[9] The inhibitors have been successfully used to find less-oxidized biosynthetic intermediates of fungal secondary metabolites, such as diacetoxyscirpenol,[10] aphidicolin,[11] nigericin,[12] betaenone B[13] and chaetoglobosin A.[14] In this paper, we extend this methodology to an investigation of the fungal metabolite alternaric acid **1**.

Results and Discussion

Isolation and Structure Elucidation of Compounds **5** *and* **6**.— During the isolation studies of alternaric acids, two new less-oxidized analogues **5** and **6** were also isolated from the same fungus as minor components.

The fungus was grown by surface culture on a Czapek Dox medium at 25 °C for 20 days. The acidic chloroform extracts of the cultural filtrates were subjected to HPLC to give compounds **5** (6.3 mg) and **6** (1.6 mg).

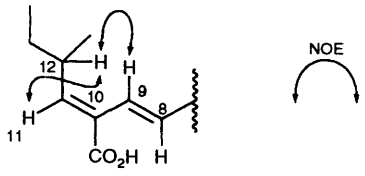

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Fig. 1 NOE experiments in compound **5** (270 MHz; $CDCl_3$)

From fast-atom-bombardment high-resolution mass spectrometry (FAB–HRMS), the molecular formula of compound **5** was determined as $C_{21}H_{30}O_6$. The UV maximum at 272 nm and the signal due to a strongly hydrogen-bonded proton at δ_H 17.90 in the ^1H NMR spectrum showed the presence of a 3-acyl-4-hydroxy-5,6-dihydro-2-pyrone structure.[1b,6,15] The ^{13}C NMR spectrum showed the presence of the four CH_3, five CH_2, six CH and six quaternary carbons. The ^1H NMR spectral data of compound **5** were similar to those of compound **3**, except for the moiety around C-10 and C-11 (Tables 1 and 2). Thus, the double doublet for the olefinic 9-H at δ_H 5.38 (*J* 15.3 and 9.2 Hz) in compound **3** was replaced by a doublet at δ_H 6.12 (*J* 15.8 Hz) in compound **5**. The triplet for the 10-H methine [δ_H 3.22 (*J* 8.9 Hz)] in compound **3** was not observed for compound **5**, and the double doublet for the 11-H methine at δ_H 3.85 (*J* 8.7 and 2.5 Hz) in compound **3** was replaced by a doublet due to the olefinic proton at δ_H 6.57 (*J* 10.6 Hz) in compound **5**. Furthermore, in the ^{13}C NMR spectrum, C-10 and C-11 of compound **5** were seen to be olefinic carbons [δ_C 128.2 (CH) and 150.4 (CH)]. These data indicate that compound **5** has a double bond between C-10 and C-11. The geometry of the C-10–C-11 double bond was determined by NOE experiments (Fig. 1). Thus, an NOE was observed between 9-H and 12-H while one was not observed between 9-H and 11-H. From these results and analysis of ^1H–^1H COSY and ^1H–^{13}C COSY data, the structure of compound **5** was determined as (10*E*)-10,11-dideoxy-10,11-dehydro-6,19-dihydroalternaric acid (Scheme 1).

Compound **6** has the molecular formula $C_{21}H_{32}O_6$ from FAB–HRMS. The ^{13}C NMR spectrum showed the presence of the four CH_3, six CH_2, six CH and five quaternary carbons. The ^1H and ^{13}C NMR spectral data of compound **6** were similar to those of compound **3**. However, a signal due to the methine group for C-11 in compound **3** was missing, and a signal due to

Table 1 ^{1}H NMR data (δ_H; $CDCl_3$) of 10-deoxy-6,19-dihydroalternaric acid **3**, (10*E*)-10,11-dideoxy-10,11-dehydro-6,19-dihydroalternaric acid **5**, 10,11-dideoxy-6,19-dihydroalternaric acid **6** and proalternaric acid I **7** [δ_H(multiplicity, *J*/Hz)]

	3[13] (500 MHz)	**5** (270 MHz)	**6** (500 MHz)	**7** (500 MHz)
4-H_a	3.06 (ddd, 15.3, 10.0, 4.4)	3.12 (m)	3.08 (m)	3.06 (ddd, 15.4, 10.1, 5.4)
4-H_b	2.96 (ddd, 15.3, 9.8, 4.9)	3.01 (m)	2.98 (ddd, 15.5, 9.7 5.6)	2.99 (ddd, 15.4, 9.8, 5.6)
5-H_a	1.63 (m)	1.65 (m)	1.51 (m)	1.64 (m)
5-H_b	1.52 (m)	1.65 (m)	1.65 (m)	1.52 (m)
6-H	1.61 (m)	1.60 (m)	1.58 (m)	1.59 (m)
7-H_a	2.07 (dt, 13.9, 6.9)	2.23 (dt, 13.9, 5.3)	2.08 (dt, 13.7, 6.4)	2.06 (dt, 13.8, 6.0)
7-H_b	1.97 (dt, 13.9, 6.9)	2.04 (dt, 13.9, 5.3)	1.94 (dt, 13.7, 6.9)	1.94 (dt, 13.8, 7.1)
8-H	5.67 (dt, 15.3, 7.1)	6.04 (dt, 15.8, 5.9)	5.56 (dt, 15.3, 7.1)	5.55 (dt, 15.3, 7.7)
9-H	5.38 (dd, 15.3, 9.2)	6.12 (dd, 15.8)	5.37 (dd, 15.3, 8.9)	5.16 (dd, 15.3, 9.3)
10-H	3.22 (t, 8.9)		3.08 (m)	2.42 (m)
11-H	3.85 (dd, 8.7, 2.5)	6.57 (d, 10.6)	1.48–1.58 (m)	3.65 (dd, 10.0, 2.5)
12-H	1.50 (m)	2.59 (m)	1.35 (m)	1.52 (m)
13-H_a	1.45 (m)	1.35 (m)	1.33 (m)	1.39 (m)
13-H_b	1.30 (m)	1.35 (m)	1.18 (m)	1.28 (m)
14-H_3	0.90 (t, 7.3)	0.86 (t, 7.3)	0.85 (t, 7.3)	0.907 (t, 7.9)
15-OH	17.89 (br s)	17.90 (br s)	17.90 (br s)	17.90 (br s)
16-H_a	2.67 (dd, 17.2, 10.0)	2.63–2.73 (m)	2.67 (dd, 17.5, 10.6)	2.66 (dd, 17.2, 10.3)
16-H_b	2.62 (dd, 17.2, 4.1)	2.63–2.73 (m)	2.62 (dd, 17.5, 4.2)	2.63 (dd, 17.2, 4.1)
17-H	4.53 (m)	4.53 (m)	4.53 (m)	4.53 (m)
18-H_3	1.46 (d, 6.3)	1.46 (d, 6.6)	1.46 (d, 6.3)	1.47 (d, 6.3)
19-H_3	0.91 (d, 6.4)	0.96 (d, 6.6)	0.91 (d, 6.5)	0.913 (d, 6.8)
20-H_a				3.81 (dd, 10.7, 7.3)
20-H_b				3.67 (dd, 10.7, 5.0)
21-H_3	0.85 (d, 7.5)	1.03 (d, 6.6)	0.84 (d, 6.8)	0.84 (d, 6.8)

Table 2 ^{13}C NMR (125 MHz; $CDCl_3$) data (δ_C) of 10-deoxy-6,19-dihydroalternaric acid **3**, (10*E*)-10,11-dideoxy-10,11-dehydro-6,19-dihydroalternaric acid **5**, 10,11-dideoxy-6,19-dihydroalternaric acid **6** and proalternaric acid I **7**

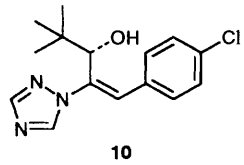
	3[5]	**5**[a]	**6**	**7**
C-1	164.5	164.6	164.3	164.3
C-2	102.9	103.3	102.9	102.9
C-3	204.7	205.0	204.7	204.7
C-4	36.2	36.4	36.4	36.6
C-5	31.3	31.5	31.3	31.3
C-6	32.7	32.9	32.8	32.8
C-7	39.5	40.8	39.5	39.7
C-8	133.9	134.6	132.7	132.2
C-9	125.6	123.2	128.8	128.9
C-10	53.7	128.2	46.7	48.3
C-11	74.5	150.4	38.8	77.5
C-12	36.4	34.7	31.6	37.2
C-13	26.7	29.7	29.6	26.8
C-14	11.8	11.8	11.2	11.9
C-15	195.0	194.9	194.9	195.0
C-16	39.4	39.4	39.4	39.4
C-17	70.4	70.3	70.3	70.3
C-18	20.6	20.6	20.6	20.6
C-19	19.3	19.3	19.2	19.3
C-20	178.1	172.6	178.7	66.7
C-21	11.7	19.8	18.4	11.6

[a] 68 MHz; $CDCl_3$.

a methylene group [δ_H 1.48–1.58 (m); δ_C 38.8] appeared for compound **6**. From the results shown above and an analysis of the ^{1}H–^{1}H COSY data, the structure of compound **6** was determined to be 10,11-dideoxy-6,19-dihydroalternaric acid (Scheme 1).

The absolute stereochemistry at C-17 in both compounds **5** and **6** was elucidated by analysis of their CD spectra. Thus, the CD spectral curves of compounds **5** and **6** have a maximum at 260 nm ($\Delta\varepsilon$ −3.3 and −3.2), similar to that of compound **3**. In an earlier paper, it was shown that the curve around 240–290 nm in the CD spectra of alternaric acids is due to the 3-acyl-4-hydroxy-5,6-dihydro-2-pyrone moiety, and the absolute configuration of C-17 can be determined by the analysis of the CD spectrum.[5] From these facts, the absolute configuration at C-17 in both compounds **5** and **6** is determined as *R*. The stereochemistry of the other stereocentres could not be determined since compounds **5** and **6** and their derivatives were not obtained as single crystals which were suitable for X-ray analysis, and the amounts of compounds isolated were not sufficient for chemical correlation. Because the alternaric acid analogues **5** and **6** have been isolated from the same fungus, it is presumed that the other stereocentres in compounds **5** and **6** have the same configurations as those of compounds **1**, **2**, **3** and **4**.

HPLC Analysis of the Cultural Extracts treated with P-450 Inhibitors.—The effect of the P-450 inhibitor S-3307D **10**[9d] on the production of alternaric acids was investigated (Table 3). Since the ε-values of the UV absorption at 274 nm in the alternaric acids are nearly the same, the values of peak area in the HPLC analysis reflect the relative amount of the metabolites.



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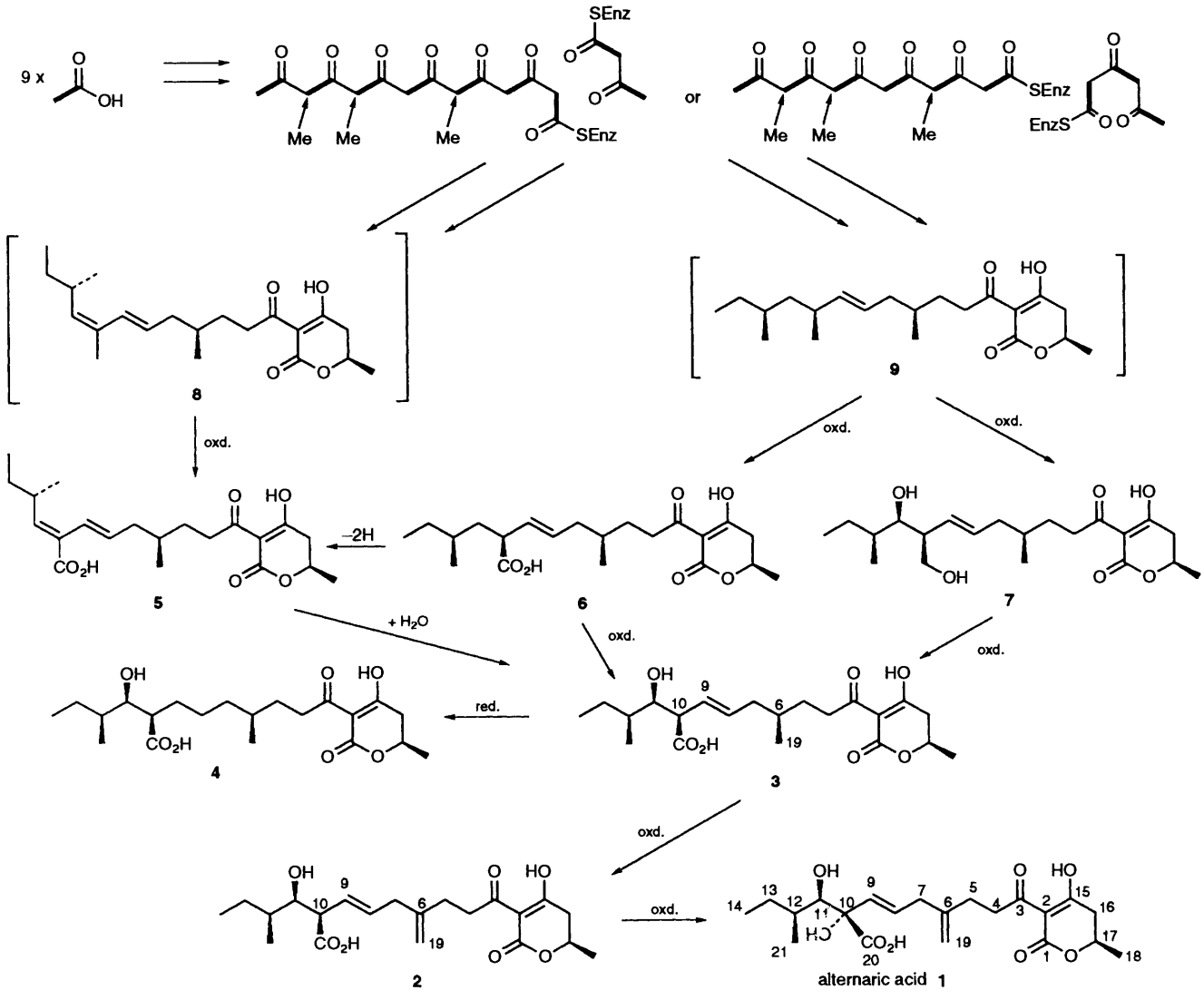
The inhibitor S-3307D **10** (1 mmol per flask) was added on the tenth day after inoculation of the culture of *A. solani* A17, which produces compounds **1**–**6**. After an additional 10 days of fermentation, the acidic chloroform extracts of the cultural filtrates were obtained and subjected to HPLC analysis. The untreated extracts were used as a control. The results in Table 3 show that treatment of the culture of *A. solani* with the inhibitor S-3307D **10**, caused a decrease in the amounts of compounds **1**, **3** and **4** obtained in the range of 1/2 to 1/3.5. In particular, the amount of compound **1** was considerably decreased (1/3.5). The total amount of alternaric acids was decreased (68%) compared with the control, while the amount of compound **5** was doubled and compound **7** was newly isolated product. These observ-



Scheme 1 Plausible biosynthetic routes to alternaric acid **1**; oxd. = oxidation, red. = reduction

Table 3 HPLC analytical data[a] of compounds **1**–**7** in the acidic extracts of cultural filtrates treated with the P-450 inhibitor S-3307D **10**

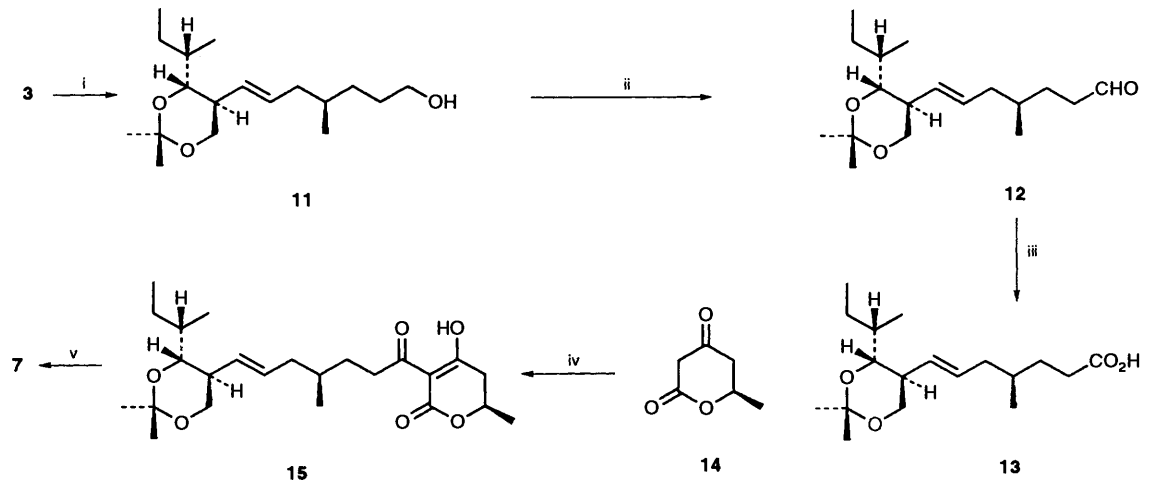
	1	2	3	4	5	6	7
Control	32.7	49.3	75.1	8.1	3.6	3.0	0.0
S-3307D	9.3	45.2	36.0	3.8	6.4	2.7	12.7

[a] The values are peak areas in HPLC analysis of compounds **1**–**7** monitored by UV absorption at 274 nm.

ations suggest that P-450 inhibitors block hydroxylations at C-10 and other oxidative steps around C-10 and C-11. The type of inhibitor used was also an important factor for the effective accumulation of less-oxidized analogues as previously reported.[14] Metyrapone[9b] and ancymidol[9e] were not as effective as S-3307D **10** for the production of compounds **5** and **7**.

Isolation and Structure Elucidation of Proalternaric Acid I **7**.—In the inhibitor experiment described above, the new metabolite **7**, which we have named proalternaric acid I, was isolated by HPLC. From field ionization high-resolution mass spectrometry (FI–HRMS), the molecular formula of component **7** was determined as $C_{21}H_{34}O_6$. The ^{13}C NMR spectrum showed the presence of the four CH_3, six CH_2, seven CH and four quaternary carbons. The compound was assumed to be a less-oxidized precursor of compound **1** since the difference between compounds **1** and **7** was the number of hydrogen and oxygen atoms. The ^1H and ^{13}C NMR spectra (Tables 1 and 2) of compound **7** were similar to those of 10-deoxy-6,19-dihydroalternaric acid **3** except for the moiety around C-20. Thus, in the ^1H NMR spectrum, the double doublet for the C-20 hydroxymethyl proton at δ_H 3.67 (J 10.7 and 5.0 Hz) and 3.81 (J 10.7 and 7.3 Hz) in compound **7** was not observed in compound **3**. Similarly, in the ^{13}C NMR spectrum, the C-20 quaternary carbon signal at δ_C 178.1 in compound **3** is replaced by a methylene carbon signal at δ_C 66.7 in compound **7**. These data indicate that the carboxy group at C-20 in compound **3** is changed into a hydroxymethyl group in compound **7**. From these observations and an analysis of the ^1H–^1H COSY data, the structure of compound **7** is deduced to be as shown in Scheme 1.

Since we failed to obtain compound **7** and derivatives as single crystals suitable for X-ray analysis, the stereochemistry of compound **7** was assigned by a chemical synthesis of compound **7** from **3** (Scheme 2). The stereochemistry of compound **3** has been determined by a combination of spectroscopic methods, chemical degradations, and interconversions in the preceding paper.[5] Compound **3** was converted into alcohol in five steps (i, Dakin oxidation; ii, diazomethane treatment; iii, *O*-acetylation;



Scheme 2 Synthesis of compound **7** from compound **3**. *Reagents and conditions:* i, 5 steps (ref. 13); ii, SO_3-Py, DMSO, Et_3N, 89%; iii, $NaClO_2$, NaH_2PO_4, 2-methylbut-2-ene, Bu^{t}OH–water, 56%; iv, DCC, DMAP, CH_2Cl_2, 50%; v, conc. H_2SO_4, MeOH, 81%.

Table 4 Results of incorporation experiments in *A. solani*. ^{13}C Chemical shifts of compounds **1** and **1a** in ^{13}C NMR spectra (125 MHz; $CDCl_3$)

	δ_c		[1-^{13}C,$^{18}O_2$]NaOAc[a]	[1-^{13}C,$^2H_3$]NaOAc[a]
	1	**1a**	**1**	**1a**
C-1	164.9	164.2	0.035 (60:40)[b]	
C-13	28.0	26.0		0.087, 0.175, 0.265 (44:5:14:37)[b,c]
C-15	194.9	194.5	0.046 (56:44)[b]	0.032 (67:33)[b,d]
C-17	70.6	70.3	0.030 (53:47)[b]	0.044, 0.084, 0.130 (45:6:13:36)[b,c]

[a] The isotope shifts are given as δ_C(ppm) upfield from the natural-abundance signal. [b] The values in parentheses are the ratio between the signal intensity of the natural-abundance peak and that of the shifted peak. [c] $CH_3:CH_2D:CHD_2:CD_3$. [d] It could not be concluded that the carbon at C-16 retains two deuteriums because the shifted signals were not clearly observed owing to the small magnitude of ^2H β-isotope shift at C-16.

iv, $LiAlH_4$ reduction; v, acetonide formation).[5] Oxidation of alcohol **11** with SO_3–pyridine yielded aldehyde **12**, which was further oxidized with sodium chlorite[16] to provide carboxylic acid **13**. We have already disclosed that the direct preparation of the 3-acyl-4-hydroxy-5,6-dihydro-2-pyrone structure from carboxylic acids and β-keto-δ-valerolactones using *N,N*-dicyclohexylcarbodiimide (DCC) and 4-dimethylaminopyridine (DMAP) in the study of the total synthesis of alternaric acid **1**.[4,17] This reaction involves a Fries type rearrangement of the *O*-enol acyl group of β-keto-δ-valerolactone towards the α-position of the δ-lactone. Using this method, carboxylic acid **13** and β-keto-δ-valerolactone **14** were converted into the dihydropyrone **15** in 50% yield. Finally, hydrolysis of the acetonide moiety in the dihydropyrone **15** furnished compound **7**, $[\alpha]_D^{24}$ −6.0 (*c* 0.36 in EtOH) {natural, $[\alpha]_D^{24}$ −4.8 (*c* 0.10 in EtOH)}, in 81% yield.

The spectroscopic data of synthetic **7** thus obtained were identical in all respects with those of natural **7**. From this result, the absolute stereochemistry of compound **7** was determined as shown. The structure and stereochemistry of the newly isolated compound **7** show that P-450 inhibitors block oxidations of the C-20 hydroxymethyl group to a carboxy group in the biosynthesis of alternaric acid **1**.

Origins of the Oxygen and Hydrogen Atoms.—From several feeding experiments,[6] the biosynthetic building units (9 acetates and 3 C_1 units) of compound **1** were established, and it was

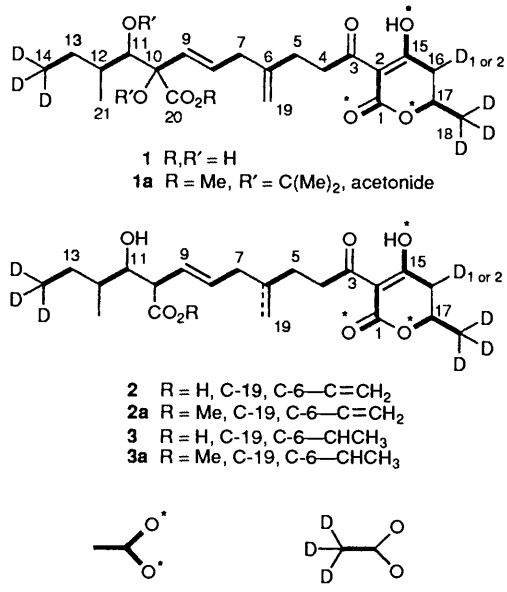
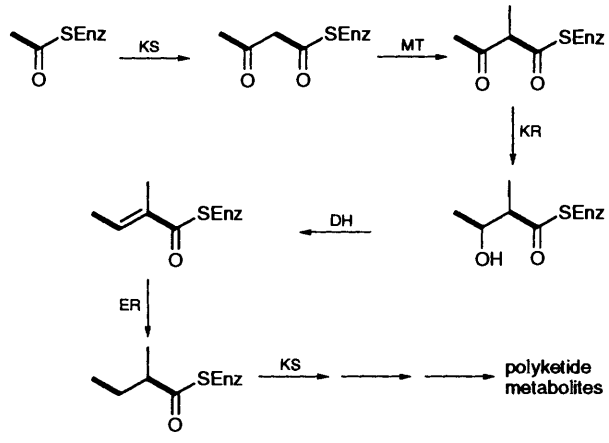


Fig. 2 Labelling of alternaric acids by sodium [1-^{13}C,$^{18}O_2$]- and [1-^{13}C,$^2H_3$]-acetate

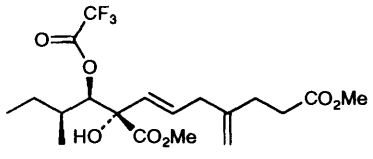
shown that compound **1** was biosynthesized by a condensation of two polyketide chains rather than from a single chain.



Scheme 3 Postulated early intermediates in the chain assembly of the polyketide synthase. KS = β-ketoacylsynthase, MT = methyltransferase, KR = β-ketoreductase, DH = dehydratase, ER = enoylreductase.

Establishing the source of the oxygen in the biosynthesis of secondary metabolites provides valuable information on the biosynthetic process.[18] Also, the fate of the hydrogen atoms on the acetate provides data for the process used for the construction of the molecular skeleton. O'Hagan *et al.* proposed that the introduction of a methyl group during the biosynthesis of fungal polyketide metabolites was controlled by several enzymatic modules and their patterns.[19] Thus, the modules consist of an enzymatic sequence from β-ketoacylsynthase (KS), methyltransferase (MT), β-ketoreductase (KR), dehydratase (DH) and enoylreductase (ER) (Scheme 3). For example, cubensic acid isolated from the fungus *Xylaria cubensis* (Mont) Fr was presumed to be biosynthesized by three modules 1–3 (module 1, KS, MT, KR, DH, ER, KS, MT, KR, DH, ER; module 2, KS, MT, KR, DH, KS, MT, KR; module 3, KS, MT, KR, DH, KS, KR).[19] Considering O'Hagan's hypothesis, the hydrogen atoms adjacent to the methyl groups are not derived from acetate. Some results of the biosynthetic studies on other fungal polyketides, chaetoglobosin A[14] and betaenone B,[20] supported this hypothesis. To test the hypothesis and to obtain pertinent data on the late biosynthetic routes, the origins of the oxygen and hydrogen atoms in the alternaric acids were investigated by the incorporation of sodium [1-^{13}C,^{18}O]- and [1-^{13}C,$^{2}H_3$]-acetate into the metabolites.

Sodium [1-^{13}C,^{18}O]acetate was administered to a culture of *A. solani.* Compounds **1**, **2** and **3** were isolated from the acidic extracts of the cultural filtrates by crystallization and HPLC as described in the preceding paper.[5] In the ^{13}C NMR spectrum of the labelled product **1**, isotopically shifted signals were observed for C-1, C-15 and C-17 as shown in Table 4 and Fig. 2. Similarly, in the ^{13}C NMR spectra of the labelled products **2** and **3**, isotopically shifted signals were also observed for C-1, C-15 and C-17, respectively (Fig. 2). On the other hand, no isotope-shifted signal at C-3 and C-11 in those of compounds **1**, **2** and **3** was detected. The small magnitudes of the ^{18}O isotope shifts at the carbons connected to hydrogen-bonded oxygens make resolution of the shifted signals difficult. Generally, it is known that attachment of an electron-withdrawing group such as an α-halogenated acetyl increases the separation of the ^{13}C–^{16}O and ^{13}C–^{18}O signals.[18,21] In order to detect the unresolved signals, the degradation product **16** of compounds **1** was prepared.



16

However, in its ^{13}C NMR spectrum, no isotopically shifted signal was observed. From these results and isolation of the less oxidative analogues, it was shown that the oxygen atoms at C-1, C-15 and C-17 in compounds **1** were derived from acetate, and that the oxygen atoms at C-10, C-11 and C-20 were probably from oxygen gas or the aqueous media. These facts are consistent with the results of inhibitor experiments. The lack of an ^{18}O label at C-3 in the degradation product **16** and the other analogues may be caused by exchange of oxygen atoms during the degradation conditions. Since, in a biosynthetic study of erythromycins, Cane *et al.* reported that the keto oxygen atom was lost at a higher level than other oxygen functional groups,[22] it is also possible that the C-3 oxygen was exchanged with the medium during the biosynthesis. The lack of an isotope-shifted signal at C-11 is not sufficient to conclude that there has been cleavage of the C-11 C–O bond. Further experiments are required to support this conclusion.

Next, we examined the incorporation of sodium [1-^{13}C,$^{2}H_3$]acetate. The alternaric acids were isolated as acetonide **1a** and methyl esters **2a** and **3a** by HPLC after methylation and acetonide formation of the acidic extracts of the cultural filtrates. In the analysis of the ^{13}C NMR spectra of compounds **1a**, **2a** and **3a**, isotopically shifted signals were seen for C-13, C-15 and C-17, respectively, as shown in Table 4 and Fig. 2. This information indicates the retention of deuteriums at C-14, C-16 and C-18 in compounds **1a**, **2a** and **3a** since the observed shifts are induced by the β-isotope effect.[18] This result is further supported by ^{2}H NMR spectroscopy for **1a** due to the observation of three signals at δ 0.85 (14-H_3), 1.44 (18-H_3) and 2.63 (16-H). The retention of three deuteriums at both C-14 and C-18 shows that alternaric acids **1**, **2** and **3** are biosynthesized from two polyketide chains (C_{14} and C_4, or C_{12} and C_6 units) rather than from a single chain.[6] The lack of retention of deuteriums at C-6, C-10 and C-12 adjacent to the methyl groups in compounds **1a**–**3a** supports O'Hagan's hypothesis. The retention of deuterium at C-16 also supports it, although exceptions are found at C-4 and C-6. Furthermore, the stereochemistries adjacent to the methyl groups in the alternaric acids suggest that the enoylreductases (ER) at each step in the biosynthesis of alternaric acid **1** closely resemble each other.

From the results of the structural elucidations of the alternaric acids, the inhibitor experiments and the incorporation experiments, a plausible biosynthetic route of alternaric acid **1** is proposed as shown in Scheme 1. Thus, compound **1** is biosynthesized *via* the putative intermediates **8** and **9**, which are then modified by stepwise introduction of oxygen atoms at C-11 and C-20 to produce compound **3**. The P-450 inhibitor, S-3307D **10**, presumably blocks these oxidative steps to provide more of the less-oxidized analogues **5** and **7**. The methyl group at C-6 in compound **3** would be converted into an *exo*-methylene group to give compound **2**. After that, a hydroxy group is introduced at C-10, α to the carboxyl group, with retention of configuration to produce alternaric acid **1**. The inhibitor probably also blocks the hydroxylation step decreasing the amount of compound **1** formed. Isolation of less-oxidized analogues is not rigorous evidence for their intermediacy. Therefore, further investigation is required to prove the proposed biosynthesis.

In conclusion, the inhibitor experiment suggests that the less-oxidized analogues are intermediates in the biosynthesis of compound **1**, and the incorporation results are consistent with this. On the basis of these results, we propose a biosynthetic pathway for alternaric acid **1**. Furthermore, cytochrome P-450 inhibitors would be useful for studies of biosynthetic pathways in other natural products since they inhibit oxidation, resulting in a less-oxidized carbon skeleton.

Experimental

General methods and instrumentation have been described previously.[5] S-3307D **10** was provided by Dr. K. Kamoshita, Sumitomo Chemical Co., Ltd. Sodium [1-^{13}C,$^{18}O_2$]- (99 atom% ^{13}C, 96% atom% ^{18}O) and [1-^{13}C,$^{2}H_3$]- (99 atom% ^{13}C, 98% atom% ^{2}H) acetate were purchased from Cambridge Isotope Lab. ^{2}H NMR spectra were measured at 76.774 MHz on a Bruker AM-500 spectrometer for $CHCl_3$ solutions in an unlocked mode (pulse width 10 μs 45°, acquisition time 2.048 s, delay time 0.5 s, 4 K data points). Natural abundance $CDCl_3$ δ_D 7.26 was used as an internal standard. $[\alpha]_D$ Values are given in units of 10^{-1} deg cm^2 g^{-1}. Ether refers to diethyl ether.

Isolation of Compounds **5** *and* **6**.—According to the preceding paper,[5] compounds **5** (6.3 mg) and **6** (1.6 mg) were isolated from the acidic extracts of the culture (7.5 dm^3) of *A. solani* by HPLC [Inertsil ODS, 10 μm, 20 × 250 mm, MeOH–0.2% H_3PO_4 (80:20)].

Compound **5**: oil; $[\alpha]_D^{26}$ −17.7 (*c* 0.52 in EtOH); CD λ_{ext}/nm ($\Delta\varepsilon$) (EtOH) 215 (+4.1), 227 (0), 238 (−2.6) and 260 (−3.3); λ_{max}(EtOH)/nm 216 (ε/dm^3 mol^{-1} cm^{-1} 14 500) and 272 (11 000); ν_{max}(NaCl)/cm^{-1} 3200, 1700, 1550, 1240 and 1060; δ_H(270 MHz; $CDCl_3$) see Table 1; δ_C(68 MHz; $CDCl_3$) see Table 2; *m/z* (FAB, negative, 3-nitrobenzyl alcohol) 377.1940 (M^- − H. $C_{21}H_{29}O_6$ requires *m/z* 377.1964).

Compound **6**: oil; $[\alpha]_D^{23}$ ~0 (*c* 0.16 in EtOH); CD λ_{ext}/nm ($\Delta\varepsilon$) (EtOH) 217 (+6.5), 231 (0) and 260 (−3.2); λ_{max}(EtOH)/nm 216 (ε/dm^3 mol^{-1} cm^{-1} 10 000) and 274 (10 000); ν_{max}(NaCl)/cm^{-1} 3200, 1710, 1570, 1260 and 1060; δ_H(500 MHz; $CDCl_3$) see Table 1; δ_C(125 MHz; $CDCl_3$) see Table 2; *m/z* (FAB, negative, 3-nitrobenzyl alcohol) 379.2126 (M^- − H. $C_{21}H_{31}O_6$ requires *m/z* 379.2121).

Semi-quantitative HPLC Analysis of Alternaric Acid Analogues obtained under Cytochrome P-450 Inhibitor Treatment.—*A. solani* A17 strain was inoculated in cultures in three 500 cm^3 flasks containing Czapek Dox medium supplemented with 0.1% yeast extract (150 cm^3), and cultured at 25 °C in the dark. 10 Days after inoculation, a solution of S-3307D **10** (146.7 mg) in EtOH (6 cm^3) was distributed equally between the three flasks (inhibitor, 1 mmol per flask). These flasks were shaken manually. After a further 10 days of fermentation, the culture filtrates were acidified with 1 mol dm^{-3} hydrochloric acid, and extracted with $CHCl_3$ (×3). The extracts were washed with 5% aq. $NaHCO_3$ (×3) and the combined alkaline aqueous layers were acidified and extracted with ethyl acetate (×3). The acidic extracts were dried over anhydrous Na_2SO_4 and then evaporated under reduced pressure. A similar experiment without the addition of the inhibitor was used as the control. The acidic extracts were individually taken up in MeOH (200 cm^3), and aliquots (10 mm^3) of each solution were subjected to analysis. Analytical conditions were as follows: UV 274 nm, flow rate 1 cm^3 min^{-1}, MeOH–0.2% aq. H_3PO_4 (73:27) for compounds **1**, **2**, **3**, **4** and **7** (t_R/min **1** 14.0, **2** 15.4, **3** 18.1, **4** 27.7, **7** 20.6), MeOH–0.2% aq. H_3PO_4 (80:20) for compounds **5** and **6** (t_R/min **5** 17.0, **6** 18.2).

Isolation of Compound **7**.—The fermentation procedure using P-450 inhibitors was described above. The acidic extracts (25.6 mg) were chromatographed by HPLC [Inertsil ODS, 10 μm, 20 × 250 mm, MeOH–0.2% H_3PO_4 (73:27)] to give proalternaric acid I **7** (1.0 mg).

Compound **7**: oil, $[\alpha]_D^{24}$ +15.1 (*c* 0.10 in EtOH); CD λ_{ext}/nm ($\Delta\varepsilon$) (EtOH) 215 (+4.9), 227 (0), 238 (−2.6) and 260 (−3.5); λ_{max}(EtOH)/nm 216 (ε/dm^3 mol^{-1} cm^{-1} 9200) and 274 (11 000); ν_{max}(NaCl)/cm^{-1} 3400, 2960, 2930, 1710, 1560, 1450, 1410, 1390, 1260, 1110 and 1060; δ_H(500 MHz; $CDCl_3$) see Table 1; δ_C(125 MHz; $CDCl_3$) see Table 2; *m/z* (FI) 383.2435 (M^+ + H. $C_{21}H_{35}O_6$ requires *m/z* 383.2434).

Aldehyde **12**.—To a stirred solution of the alcohol **11**[5] (16.6 mg, 0.0557 mmol) and triethylamine (0.25 cm^3) in DMSO (0.8 cm^3) was added SO_3–pyridine (94 mg, 0.588 mmol) portionwise. After being stirred for 1 h at room temperature, the reaction mixture was quenched by the addition of aq. $NaHSO_4$ (pH 2 ~ 3), and then extracted with ethyl acetate (×3). The combined organic layers were dried ($MgSO_4$), and then evaporated under reduced pressure. Flash column chromatography of the residue on silica gel with hexane–ether (8:2) yielded the aldehyde **12** (14.6 mg, 89%) as an oil, $[\alpha]_D^{22}$ +36.7 (*c* 1.46 in $CHCl_3$); ν_{max}(NaCl)/cm^{-1} 1730, 1260, 1230 and 1070; δ_H(270 MHz; $CDCl_3$) 9.76 (1 H, br s, 3-H), 5.50 (1 H, dt, *J* 15.2 and 7.3, 8-H), 5.07 (1 H, dd, *J* 15.2 and 9.2, 9-H), 3.64 (3 H, m, 11-H and 20-H_2), 2.43 (3 H, m, 4-H_2 and 10-H), 2.02 (1 H, dt, *J* 13.9 and 6.3, 7-H_a), 1.87 (1 H, dt, *J* 13.9 and 6.9, 7-H_b), 1.66 (1 H, m, 6-H), 1.21–1.56 (5 H, m, 5-H_2, 12-H and 13-H_2), 1.42 and 1.36 (each 3 H, each s, acetonide Me_2) and 0.86 (9 H, m, 14-H_3, 19-H_3 and 21-H_3); δ_C(68 MHz; $CDCl_3$) 202.6, 131.9, 128.3, 97.9, 74.2, 64.8, 41.6, 41.1, 39.9, 35.9, 32.5, 29.6, 28.3, 26.4, 19.1, 18.9, 12.5 and 11.9; FI–MS *m/z* 297 (M^+ + H); EI–HRMS *m/z* 221.1913 (M^+ − $C_3H_7O_2$. $C_{15}H_{25}O$ requires *m/z* 221.1905).

Carboxylic Acid **13**.—To a stirred solution of the aldehyde **12** (14.0 mg, 0.0473 mmol), 2-methylbut-2-ene (44 mm^3, 0.399 mmol) and monobasic sodium phosphate (11.2 mg, 0.0718 mmol) in a mixture of *tert*-butyl alcohol (0.7 cm^3) and water (0.2 cm^3) was added sodium chlorite (86% purity; 29.2 mg, 0.323 mmol) portionwise at 10 °C. After being stirred at 10 °C for 40 min, water was added. The resulting mixture was acidified with 0.1 mol dm^{-3} hydrochloric acid, and extracted with ethyl acetate (×3). The combined organic layers were dried ($MgSO_4$) and then evaporated under reduced pressure. Flash column chromatography of the residue on silica gel with $CHCl_3$–MeOH (98:2) yielded the carboxylic acid **13** (8.6 mg, 58%) as an oil, $[\alpha]_D^{22}$ +39.5 (*c* 0.86 in $CHCl_3$); ν_{max}(NaCl)/cm^{-1} 3100, 1710, 1260, 1230 and 1070; δ_H(270 MHz; $CDCl_3$) 5.50 (1 H, dt, *J* 15.2 and 7.3, 8-H), 5.07 (1 H, dd, *J* 15.2 and 8.9, 9-H), 3.64 (3 H, m, 11-H and 20-H_2), 2.43 (3 H, m, 4-H_2 and 10-H), 2.01 (1 H, dt, *J* 13.9 and 5.9, 7-H_a), 1.87 (1 H, dt, *J* 13.9 and 6.6, 7-H_b), 1.68 (1 H, m, 6-H), 1.24–1.58 (5 H, m, 5-H_2, 12-H and 13-H_2), 1.42 and 1.35 (each 3 H, each s, acetonide Me_2) and 0.86 (9 H, m, 14-H_3, 19-H_3 and 21-H_3); δ_C(68 MHz; $CDCl_3$) 202.6, 131.9, 128.3, 97.9, 74.2, 64.8, 41.6, 41.1, 39.9, 35.9, 32.5, 29.6, 28.3, 26.4, 19.1, 18.9, 12.5 and 11.9; FI–MS *m/z* 313 (M^+ + H); EI–HRMS *m/z* 237.1861 (M^+ − $C_3H_7O_2$. $C_{15}H_{25}O_2$ requires *m/z* 237.1854).

Dihydropyrone **15**.—A solution of carboxylic acid **13** (8.2 mg, 0.0263 mmol), β-keto-δ-valerolactone **14**[4] (3.8 mg, 0.0297 mmol), DMAP (0.8 mg, 0.0066 mmol) and DCC (40 mg, 0.194 mmol) in dry CH_2Cl_2 (1.0 cm^3) was stirred at room temperature for 19 h. The reaction mixture was filtered and the filtrates were washed with sat. aq. NH_4Cl, dried ($MgSO_4$), and then evaporated under reduced pressure. Flash column chromatography of the residue on silica gel with $CHCl_3$ yielded the dihydropyrone **15** (4.9 mg, 42%) as an oil, $[\alpha]_D^{23}$ ~0 (*c* 0.29 in $CHCl_3$); ν_{max}(NaCl)/cm^{-1} 3400, 1710, 1460, 1260, 1200 and 1060; δ_H(270 MHz; $CDCl_3$) 17.90 (1 H, br s, 15-OH), 5.51 (1 H, dt, *J* 15.2 and 7.3, 8-H), 5.07 (1 H, dd, *J* 15.2 and 8.9, 9-H), 4.52 (1 H, m, 17-H), 3.59–3.67 (3 H, m, 11-H and 20-H_2), 3.08 (1 H, m, 4-H_a), 2.97 (1 H, m, 4-H_b), 2.58–2.66 (2 H, m, 16-H), 2.42 (1 H, m, 10-H), 2.04 (1 H, m, 7-H_a), 1.88 (1 H, m, 7-H_b), 1.20–1.68 (6 H, m, 5-H_2, 6-H, 12-H and 13-H_2), 1.46 (3 H, d, *J* 5.9, 18-H_3), 1.42 and 1.35 (each 3 H, each s, acetonide Me_2) and 0.83–0.90 (9 H, m, 14-H_3, 19-H_3 and 21-H_3); FI–MS *m/z* 423 (M^+ + H); EI–HRMS *m/z* 347.2189 (M^+ − $C_3H_7O_2$. $C_{21}H_{31}O_4$ requires *m/z* 347.2222).

Synthesis of Compound **7**.—To a solution of dihydropyrone **15** (4 mg, 0.0095 mmol) in MeOH (3 cm^3) was added conc. sulfuric acid (10 mm^3). The solution was stirred at room temperature overnight, and then evaporated under reduced pressure to 0.2–0.3 cm^3. Water (20 cm^3) was added to it, and the mixture extracted with ethyl acetate (×3). The combined organic layers were washed with brine, dried ($MgSO_4$), and then evaporated under reduced pressure. The residue was subjected to PTLC, developed with $CHCl_3$–MeOH (9:1), to yield compound **7** (3.6 mg, 81%) as an oil, $[\alpha]_D^{22}$ −6.0 (*c* 0.36 in EtOH).

Feeding Experiments with Sodium [1-^{13}C,$^{18}O_2$]- *and* [1-^{13}C,$^2H_3$]-*Acetate*.—The fungus was inoculated in six 500 cm^3 flasks containing Czapek Dox medium (0.1% yeast extract)

(150 cm^3). 10 Days after inoculation in the dark at 25 °C, an aqueous solution (12.6 cm^3) of [1-^{13}C,$^{18}O_2$]acetate (300 mg), which had been filtered through a sterilized microfilter (0.2 μm), was equally distributed between the six 500 cm^3 flasks. After a further 10 days of incubation, the culture filtrates were worked up as described in the preceding paper.[5] Crystallization and HPLC of the extracts (409.3 mg) afforded compounds **1** (38 mg), **2** (52 mg) and **3** (101 mg).

Similarly, the feeding experiment with [1-^{13}C,$^{2}H_3$]acetate (300 mg) afforded the acidic extracts (38 mg). Without purification, the acidic extracts were treated with MeOH–sulfuric acid.

To a solution of the acidic extracts (36 mg) in MeOH (1.5 cm^3) was added conc. sulfuric acid (30 mm^3) at 0 °C. After being stirred at room temperature for 48 h, the reaction mixture was poured into ice–water, and extracted with ethyl acetate (× 3). The combined organic layers were washed with brine, dried ($MgSO_4$), and then evaporated under reduced pressure to give a crude product. The product was employed without further purification in the subsequent step.

To a solution of the product in acetone (1.5 cm^3) was added conc. sulfuric acid (20 mm^3) at 0 °C. The mixture was stirred at room temperature for 48 h, and then poured into ice–water and extracted with ethyl acetate (× 3). The combined organic layers were washed with brine, dried ($MgSO_4$), and then evaporated under reduced pressure. The residue (38 mg) was chromatographed by HPLC [Inertsil ODS, 10 μm, 20 × 250 mm, MeOH–0.2% H_3PO_4 (80 : 20)] to give compounds **1a**[4] (4.2 mg), **2a** (3.5 mg) and **3a** (6.8 mg).

Compound **1a**: δ_D(76.8 MHz; $CDCl_3$) 2.68 (16-H_2), 1.44 (18-H_3) and 0.85 (14-H_3).

Compound **2a**: oil, $[\alpha]_D^{23}$ +8.8 (*c* 0.53 in EtOH); CD λ_{ext}/nm (Δε) (EtOH) 215 (+9.4), 232 (0) and 258 (−2.7); λ_{max}(EtOH)/nm 215 (ε/dm^3 mol^{-1} cm^{-1} 10 000) and 275 (10 000); ν_{max}(NaCl)/cm^{-1} 3450, 1710, 1560, 1260 and 1060; δ_H(500 MHz; $CDCl_3$) 17.83 (1 H, br s, 15-OH), 5.65 (1 H, dt, *J* 15.3 and 7.0, 8-H), 5.42 (1 H, dd, *J* 15.3 and 9.3, 9-H), 4.80 (1 H, br s, 19-H_a), 4.76 (1 H, br s, 19-H_b), 4.53 (1 H, m, 17-H), 3.85 (1 H, dd, *J* 8.9 and 2.2, 11-H), 3.72 (3 H, s, OMe), 3.22–3.26 (1 H, m, 4-H_a), 3.24 (1 H, t, *J* 9.2, 10-H), 3.13 (1 H, m, 4-H_b), 2.79 (2 H, d, *J* 6.9, 7-H_2), 2.68 (1 H, dd, *J* 17.4 and 11.2, 16-H_a), 2.63 (1 H, dd, *J* 17.4 and 4.2, 16-H_b), 2.35 (2 H, m, 5-H_2), 1.62 (1 H, m, 12-H), 1.47 (3 H, d, *J* 6.3, 18-H_3), 1.44 (1 H, m, 13-H_a), 1.30 (1 H, m, 13-H_b), 0.90 (3 H, t, *J* 7.4, 14-H_3) and 0.85 (3 H, d, *J* 6.6, 21-H_3); δ_D(76.8 MHz; $CHCl_3$) 2.63 (16-H_2), 1.44 (18-H_3) and 0.87 (14-H_3); FD–MS *m/z* 409 (M^+ + H).

Compound **3a**: oil, $[\alpha]_D^{23}$ +23.0 (*c* 0.27 in EtOH); CD λ_{ext}/nm (Δε) (EtOH) 213 (+10.7), 232 (0) and 252 (−3.0); λ_{max}(EtOH)/nm 215 (ε/dm^3 mol^{-1} cm^{-1} 10 000) and 275 (10 000); ν_{max}(NaCl)/cm^{-1} 3450, 1710, 1570, 1260 and 1060; δ_H(500 MHz; $CDCl_3$) 17.90 (1 H, br s, 15-OH), 5.61 (1 H, dt, *J* 15.4 and 7.1, 8-H), 5.35 (1 H, dd, *J* 15.4 and 9.4, 9-H), 4.53 (1 H, m, 17-H), 3.84 (1 H, d, *J* 9.9, 11-H), 3.71 (3 H, s, OMe), 3.20 (1 H, t, *J* 9.2, 10-H), 3.09 (1 H, m, 4-H_a), 2.97 (1 H, m, 4-H_b), 2.67 (1 H, dd, *J* 17.3 and 10.7, 16-H_a), 2.62 (1 H, dd, *J* 17.3 and 3.9, 16-H_b), 2.08 (1 H, m, 7-H_a), 1.93 (1 H, m, 7-H_b), 1.48–1.66 (4 H, m, 5-H_2, 6-H and 12-H), 1.46 (3 H, d, *J* 6.2, 18-H_3), 1.42 (1 H, m, 13-H_a), 1.30 (1 H, m, 13-H_b), 0.900 (3 H, d, *J* 6.3, 19-H_3), 0.899 (3 H, t, *J* 7.4, 14-H_3) and 0.84 (3 H, d, *J* 6.6, 21-H_3); δ_D(76.8 MHz; $CHCl_3$) 2.61 (16-H_2), 1.41 (18-H_3) and 0.83 (14-H_3); FD–MS *m/z* 411 (M^+ + H).

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